

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
 US Department of Commerce
 United States Patent and Trademark
 Office, PCT
 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 22 January 2001 (22.01.01)	
International application No. PCT/US00/12638	Applicant's or agent's file reference PCT 20455
International filing date (day/month/year) 10 May 2000 (10.05.00)	Priority date (day/month/year) 14 May 1999 (14.05.99)
Applicant BLUE, Jeffrey, T.	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

08 November 2000 (08.11.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

Form PCT/IB/2000/1
 (2000 Geneva 20) Switzerland

Form PCT/IB/2000/1

Form PCT/IB/2000/1

Form PCT/IB/2000/1

Form PCT/IB/2000/1

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 09 FEB 2001

WIPO

PCT

Applicant's or agent's file reference PCT 20455	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/12638	International filing date (day/month/year) 10 MAY 2000	Priority date (day/month/year) 14 MAY 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant MERK & CO., INC.		

1 This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2 This REPORT consists of a total of 4 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3 This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Name and mailing address of the IPEA US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

LAURIE SCHEINER

Examination No. 703-305-3231

Telephone No. 703-305-3231

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/12638

I. Basis of the report

1. With regard to the **elements** of the international application:*

- ☒ the international application as originally filed
- ☒ the description
pages 1-12, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the claims
pages 13-14, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the drawings:
pages 1-6, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item

These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in printed form
- ☐ filed together with the international application in computer readable form
- ☐ furnished subsequently to this Authority in written form
- ☐ furnished subsequently to this Authority in computer readable form
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4 ☒ The amendments have resulted in the cancellation of _____☒ the drawings. sheets 4-6 NONE5 ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)) **

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 17 are referred to in this report as "originally filed" and are not annexed to this report, but they are mentioned in the amendments (Rule 70.2(c)).

** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/12638

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 statement

Novelty (N)

Claims 1-17

YES

Claims NONE

NO

Inventive Step (IS)

Claims 1-17

YES

Claims NONE

NO

Industrial Applicability (IA)

Claims 1-17

YES

Claims NONE

NO

2 citations and explanations (Rule 70.7)

Claims 1-17 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a method for assaying the virulence of a particular virus by the employment and subsequent measurement of caspase 3.

----- NEW CITATIONS -----

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/12638

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C12Q 1/02, 1/70; G01N 33/573; A61K 49/00; A01N 63/00 and US Cl.: 435/5, 7.4, 29; 424/9.2, 93.6

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/02, 1/70, G01N 33/573, A61K 49/00, A01N 63/00		(11) International Publication Number: WO 00/70081
A1		(43) International Publication Date: 23 November 2000 (23.11.00)
(21) International Application Number: PCT/US00/12638	(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 10 May 2000 (10.05.00)		
(30) Priority Data: 60 134,163 14 May 1999 (14.05.99) US	Published <i>With international search report.</i>	
(71) Applicant (for all designated States except US): MERCK & CO., INC. [US US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).		
(72) Inventor; and (75) Inventor/Applicant (for US only): BLUE, Jeffrey, T. [US US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).		
(74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).		

(54) Title: DETECTION OF VIRAL STABILITY

(57) Abstract

Viral induction of caspase 3 activity was found to provide a reliable measure of viral activity. Assaying viral induction of caspase 3 activity can be used, for example, in methods for measuring viral potency and stability, and for evaluating the stability of a virus in different formulations.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AI	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MS	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NF	Norfolk	XX	Unknown
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	UK	Scotland	SE	Sweden		

TITLE OF THE INVENTION
DETECTION OF VIRAL STABILITY

FIELD OF THE INVENTION

5 The present invention pertains to assays for measuring viral potency and stability and for evaluating the stability of a virus in different formulations.

BACKGROUND OF THE INVENTION

10 The references cited herein are not admitted to be prior art to the claimed invention.

 In developing live virus vaccines it is important to determine viral potency and stability to assure adequate immunization. The plaque assay is a common method for determining viral potency and stability. The plaque assay is often used to examine the effect of different storage conditions on viral stability.

15 The plaque assay involves inoculating viruses onto a cell sheet at dilutions determined by the expected (estimated) potency. The plates containing the infected cells are incubated for a specified length of time and then stained. Viral infection results in plaque formation, or areas of dead or detached cells. Counting the number of plaques gives the corresponding plaque-forming units (PFU), a measure of
20 potency. Viral stability is determined by examining the change in PFU over time.

SUMMARY OF THE INVENTION

 Viral induction of caspase 3 activity was found to provide a reliable measure of viral activity. Assaying viral induction of caspase 3 activity can be used, for
25 example, in methods for measuring viral potency and stability, and for evaluating the stability of a virus in different formulations.

 Thus, a first aspect of the present invention describes a method of assaying the potency and stability of a virus by measuring viral induction of caspase 3 activity. The method involves infecting a plurality of cells susceptible to caspase 3 induction
30 with the virus and measuring caspase 3 activity as an indication of viral potency and stability. A plurality of cells is a population of the same strain of cells.

susceptible to caspase 3 induction, with a virus in different test formulations. The

strain of cells is provided as different pluralities of cells where each plurality of cells is infected with the virus. Induction of caspase 3 activity produced from virus stored in the different formulations is measured to identify the formulations that stabilize the virus.

5 Other features and advantages of the present invention are apparent based on the descriptions provided herein. The examples provided herein illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology for
10 practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b illustrate induction times for caspase 3 activity using lyophilized virus and liquid bulk virus (*i.e.*, a concentrated lot of clarified virus). The
15 RFU (relative fluorescent unit) value was adjusted by subtracting background signal (adjusted RFU). Figure 1a illustrates an induction time using lyophilized mumps virus ("MuV"), lyophilized rubella virus ("RbV"), and lyophilized measles virus ("MeV"). Figure 1b illustrates an induction time using bulk measles virus.

Figures 2a and 2b illustrate a linear range for induction of caspase 3 activity.
20 Figure 2a provides results using measles virus. Figure 2b provides results using mumps virus.

Figure 3 illustrates measles virus induction variation in various samples. "Liquid" indicates a non-lyophilized frozen aliquot. "D0" indicates a lyophilized non-incubated sample stored at -70°C. The one-week sample was initially lyophilized
25 and stored at 37°C for one week. "A", "C", "D" and "F" refer to different formulations of the vaccine.

Figure 4 illustrates a comparison of the plaque assay and the caspase 3 assay. Activity was measured using measles virus stored in different environments. "Liq" indicates a non-lyophilized frozen aliquot. "D0" indicates a lyophilized, non-
30 incubated sample stored at -70°C. "D7" indicates a lyophilized sample incubated at 37°C for one week. "A", "B", "C" and "D" refer to different formulations.

Liq" indicates a non-lyophilized frozen aliquot. "D0" indicates a lyophilized sample

incubated sample stored at -70°C . "D7" indicates a lyophilized sample incubated at 37°C for one week. "A", "B", "C" and "D" refer to different vaccine formulations being tested.

Figure 6 illustrates a comparison of the plaque assay and the caspase 3 assay.

- 5 Viral activity was measured using rubella virus stored in different environments. "Liq" indicates non-lyophilized frozen aliquot. "D0" indicates a lyophilized, non-incubated sample stored at -70°C . "D7" indicates lyophilized sample incubated at 37°C for one week. "H", "I", "J", "K" and "L" refer to different formulations of the vaccine.

10

DETAILED DESCRIPTION OF THE INVENTION

- Measuring viral induction of caspase 3 activity was found to provide a reliable measure of viral potency and stability. Useful properties of measuring viral induction of caspase 3 activity include: induction of caspase 3 activity is achieved by different
15 viruses including measles, mumps and rubella viruses; induction of caspase 3 activity is reproducible; induction of caspase 3 activity is sensitive to viral dilutions; induction of caspase 3 activity is sensitive to sample differences; and induction of caspase 3 activity correlates with the plaque assay. Thus, the caspase 3 assay can be applied to different formulations and a variety of viruses. Additionally, caspase 3 activity is not
20 substantially effected by freezing and thawing, can be specifically detected, and is quenchable. These advantages allow the assay to be easily implemented for routine laboratory handling.

- Another advantage of the caspase 3 assay in time. When measuring viral stability, the caspase 3 assay can be performed quicker than the plaque assay. The
25 standard plaque assay takes approximately seven to ten days, but the caspase 3 assay can be performed in about half that time.

Caspase 3

- Caspase 3 (also known as CPP32 or apopain) is a member of the caspase
30 family of proteases. Caspases are induced by apoptosis, which is an active process of cellular suicide. (*Thompson, Science* 267:1456-1462, 1995; *Nicholson, Nature*

Development 5:105-111, 1995; Sadzot-Delvaux, *et al.*, *Journal of General Virology* 76:2875-2879, 1995; Esolen, *et al.*, *Journal of Virology* 69:3955-3958, 1995; and Ito, *et al.*, *FEMS Immunology and Medical Microbiology* 15:115-122, 1996.) However, certain viral proteins have been indicated to block apoptosis, such as Baculovirus protein p35 which inhibits caspase 3. (Lincz, *Immunology and Cell Biology* 76:1-19, 1998.)

Caspase 3 is a cysteine protease synthesized from a pro-enzyme by cleavage at an Asp residue to form an active protease. The active caspase 3 protease also cleaves at Asp residues. The specific recognition sequence for caspase 3 is the peptide Asp-Glu-Val-Asp. (Fernandes-Alnemri, *et al.*, *Journal of Biological Chemistry* 269:30761-30764, 1994; Nicholson, *et al.*, *Nature* 376:37-43, 1995; Srinivasula, *et al.*, *Journal of Biological Chemistry* 271:27099-27106, 1996; Schlegel, *et al.*, *Journal of Biological Chemistry* 271:1841-1844, 1996; and Casciola-Rosen, *et al.*, *J. Exp. Med.* 183:1957-1964, 1996; each of these references is hereby incorporated by reference herein.)

Selection of Appropriate Viruses and Cells

Suitable virus and cells for use in the present invention can be selected based upon the susceptibility of a particular cell to caspase 3 induction when infected with a particular virus. The ability of a virus to infect a particular cell can readily be determined, as can the ability of the virus to induce caspase 3 activity. Examples of procedures that can be used to measure the suitability of a cell and a virus are described in the Example section provided below using measles, mumps and rubella virus. Other cells (*e.g.*, MRC-5) and viruses (*e.g.*, varicella-zoster) are expected to be useful in the present invention.

Measuring Caspase 3 Activity

"Caspase 3 activity" refers to enzymatic activity able to cleave the caspase 3 substrate Asp-Glu-Val-Asp ("DEVD"). Such activity is known to be produced by caspase 3 and at least one related enzyme, Mch3a. (Fernandes-Alnemri, *et al.*, *Cancer Research* 55:6045-6052, 1995, which is hereby incorporated by reference

can be measured using techniques well known in the art. Preferably, caspase 3

activity is measured using colorimetric or fluorimetric labeled substrates. More preferably, the employed substrate is DEVD linked to a colorimetric or fluorimetric moiety. Examples of such a moiety include the colorimetric moiety *p*-nitroanilide ($\lambda_{\text{max}} = 505 \text{ nm}$) and the fluorimetric moiety 7-amino-4-trifluoromethyl coumarin ("AFC", $\lambda_{\text{max}} = 400 \text{ nm}$). (Zhang, *et al.*, in *Apoptosis Detection and Assay Methods*, pages 7-14, Eds. Zhu and Chun, *BioTechniques Books*, 1998, both of which are hereby incorporated by reference herein). Colorimetric and fluorimetric labeled substrates can be employed using procedures such as those described by Zhang, *et al.*, in *Apoptosis Detection and Assay Methods*, pages 7-14, Eds. Zhu and Chun, *BioTechniques Books*, 1998; and ApoAlert™ CPP32/Caspase-3 Assay Kits User Manual (PT3083-1), CLONTECH Laboratories, Inc. 1998; both of which are hereby incorporated by reference herein.

The caspase 3 assay is preferably employed on viral vaccine samples either in liquid or lyophilized form. For example, lyophilized viral samples can be reconstituted, diluted, and plated onto a cell sheet; the samples are incubated, the cells are lysed, and the cellular lysate collected and frozen at -70°C ; after thawing on wet ice (about 5°C) removal of cellular debris by centrifugation occurs, the supernatant is removed to a new tube containing reaction buffer, and the DEVD-AFC substrate is incubated with the cellular supernatant.

EXAMPLES

Examples are provided below to further illustrate different features and advantages of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Methods and Materials

Methods and materials generally used in growing cells, inducing caspase 3 activity, and measuring caspase 3 activity to obtain the results provided below were as follows:

Growth of Vero Cells and RK-13 Cells

neomycin at 25°C with 5% CO_2 . The flasks were then decanted and 2 ml of 1x

trypsin-ethylene diamine tetra acetic acid (EDTA) (Gibco/BRL cat# 25200-056) was added to the flasks to neutralize the media. The trypsin was decanted and another 2 mls of trypsin was added and incubated for 10 to 15 minutes at 35°C with 5% CO₂. Cells were then washed to the bottom of the flask with 20 ml of M199 containing 10% FBS and 0.5% neomycin.

Cells were plated into 24 well plates at 5.4×10^4 cells/ml. Each well received 2 ml of the diluted cells (diluted into M199 containing 10% FBS and 0.5% neomycin). The plates were incubated at 35°C for 2 days and then used for caspase 3 induction. The growth of RK-13 cells is the same as Vero cells with the exception of the media. RK-13 cells were grown in MEM media (Gibco/BRL) containing 10% FBS and 0.5% neomycin. The cells were plated at a density of 6.0×10^4 cells /ml, 2 ml per well.

Induction of Caspase 3 Activity

Generally, Vero plates were used for measles virus and mumps virus infections, while RK-13 cells were used for the rubella virus infections. Lyophilized formulations of a combination of measles, mumps, and rubella vaccine were used. Lyophilized formulations were reconstituted with 0.7 ml dH₂O. Frozen liquid samples were thawed at room temperature prior to use. Following reconstitution or liquid thawing, the samples were diluted 1:2, 1:5, 1:10 or 1:20 in their respective diluents. The diluents contained antibodies against the viruses not being assayed for among mumps, measles, and rubella viruses (*e.g.*, when assaying for measles virus antibodies for mumps and measles virus were used).

Cells (2.0×10^5) were infected with 50 µl of the diluted samples. Each sample was used to infect 6 wells on a 24 well plate. Plates were then incubated at 35°C with 5% CO₂ for 1 hr for plates to be assayed for mumps and rubella viruses and for 2 hrs for plates to be assayed for measles virus. During this attachment phase, the plates were rocked every 15 minutes to ensure proper coverage of the cell sheet with media. Following the attachment phase, the cells were overlaid with 1 ml of M199 media containing 10% FBS and 0.5% neomycin for the Vero cells and 1 ml of MEM containing 10% FBS and 0.5% neomycin for the RK-13 cells. The plates were then

Caspase 3 Assay

Following the induction period, media was aspirated from the wells. Thirty microliters of chilled cell lysis buffer (Clontech: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100) was added per well. The samples were incubated on ice for 10 minutes and then two wells were pooled together into one microfuge tube. Samples were either frozen on dry ice and stored at -70°C until time of analysis or were assayed immediately.

The samples were spun in at 12,000 rpm for 5 minutes at 4°C. Supernatant (50 µl) was removed to a new microfuge tube and 50 µl of 2x Reaction Buffer (Clontech: 100 mM HEPES, pH 7.4, 150 mM NaCl, 0.2% CHAPS) containing 7 mM dithiothreitol (DTT) was added. Five microliters of the 1 mM DEVD-AFC substrate was added to the reaction and vortexed briefly. The samples were then incubated for 1 hr at 37°C in a circulating water bath. Following the incubation, 20 µl of 10% H₃PO₄ was used to quench the reaction. Quenched reactions (100 µl) were added to a 96 well plate and read in a TECAN fluorescent plate reader (excitation: 390 nm, emission: 480 nm, gain: 60).

Results

Detection of Caspase 3 Activity Induced by Measles, Mumps and Rubella Viruses

The ability of measles, mumps, and rubella viruses to induce caspase 3 activity in cells was confirmed using Vero and RK-13 cells. Caspase 3 activity was measured as described above in Methods and Materials. Vero cells were induced with measles virus, mumps virus, and rubella virus, while RK-13 cells were induced with rubella virus. The results are shown in Table 1

Table 1

	Induced	Non-Induced
Measles Virus	12861	1210
Mumps Virus	20085	1286

80 CONTROLS.

To ensure that the observed signal was due to the virus, one set of Vero cells were infected with measles virus and another set of Vero cells were infected with a placebo. The placebo produced an adjusted RFU of 50-500, while measles virus produced an adjusted RFU of about 7,750. These results demonstrate that significant changes in caspase 3 activity are due to viral infection rather than a formulation component.

Effects of Freeze-Thawing on Caspase 3 Activity

To allow an assay to be run at various times after infected, samples may be frozen and later thawed at a convenient time. To determine if freezing and thawing affects caspase 3 activity, samples were frozen on dry ice and stored at -70°C following cell lysis. The samples were then thawed and assayed with a set of samples that were not frozen. Non-frozen cells provided an adjusted RFU of 2,167 while frozen cells produced an adjusted RFU of 2,175.5. Thus, freeze-thawing does not appear to effect caspase 3 activity. Accordingly, many samples can be run and then stored for various lengths of time allowing the actual assay to be run at a later time.

Determination of the Optimal Time Required for Viral Bulk Incubation

The time required to induce caspase 3 activity with various samples can differ depending on the virus and multiplicity of infection (MOI). To determine preferred times for measles, mumps and rubella virus infection, Vero cells or RK-13 cells were infected with (1) measles virus bulk and (2) different lyophilized measles virus, mumps virus, and rubella virus. Vero cells were infected with mumps and measles, while RK-13 cells were infected with rubella virus. A long-term study was initiated with time points collected at 24, 46, 72, and 96 hours for bulk samples and 26, 47, 75, 94, 95, 97 and 100 hours for lyophilized samples.

The results of different points are presented in Figures 1a and 1b. All three viruses in lyophilized formulations show a peak signal around 95 hours (Figure 1a). The optimal signal observed for the infection with measles virus bulk was around 46 hours (Figure 1b). This difference in overall time is expected since a bulk sample contains a much higher titer and infects more cells upon inoculation, thereby

Linearity of the Caspase 3 Enzyme Response from Cells induced with Measles Virus and Mumps Virus

When investigating and comparing enzyme activity, it is important to collect data found within the linear range of the assay. Following induction with measles and mumps viruses, the samples were assayed and data collected over a one-hour period for the measles virus and an hour and a half for mumps virus. The data presented in Figure 2a (measles virus) shows the assay is linear for at least one hour, and the data presented in Figure 2b (mumps virus) show the assay is linear for at least 75 minutes. Thus, using a one-hour incubation period for caspase 3 enzyme activity will ensure the data collected will be meaningful.

Determination of Non-Specific Substrate Cleavage

A concern with using a non-purified cell lysate is the effect of non-specific substrate cleavage derived from other proteases found within the lysate. Using a specific inhibitor for caspase 3, DEVD-CHO, the caspase 3 assay was run and analyzed. If non-specific cleavage were occurring in the assay, the addition of the inhibitor would not completely stop the substrate from being cleaved. The results shown in Table 2 indicate that adding 1 μ M DEVD-CHO inhibitor eliminates the RFU signal.

TABLE 2

Virus	- Inhibitor (RFU)	+ Inhibitor (RFU)
Measles Virus	2538	6
Rubella Virus	3205	10
Mumps Virus	3074	0

RFU was adjusted to take background into account.

Samples without the caspase 3 inhibitor show a ~3,000 fold increase over the

The Effects of Quenching Caspase 3 Assay using 10% H₃PO₄

To ensure consistent data when using enzymes, it is useful to be able to quench the reaction. Quenching can be used to ensure that each sample is incubated for the same amount of time. It is useful to examine the quenching conditions to ensure that the quench is not having an effect on the RFU signal that is being generated. In some instances, quenching a fluorescent peptide will provide interference on the overall signal making data interpretation difficult.

Following a one hour incubation period, samples were quenched with 20 µl of 10% H₃PO₄ and analyzed on the TECAN Plate reader. Data were compared with that obtained from samples that did not undergo quenching. The results are shown in Table 3.

TABLE 3

	Not Quenched (RFU)	Quenched (RFU)	Non-Induced Not Quenched (RFU)	Non-Induced Quenched (RFU)
Measles Virus	9331	5069	1046	508

The results indicate the ratio of the non-induced samples to the induced samples is the same. Thus, quenching the reactions does not effect the relative signal intensity.

Reproducibility of the Caspase 3 Assay

Using samples induced by measles virus, mumps virus, or rubella virus, the reproducibility of measuring caspase 3 activity was examined by using three vials of the same sample. The data presented in Table 4 show that the assay is reproducible and very consistent.

TABLE 4

	Sample 1 (RFU)	Sample 2 (RFU)	Sample 3 (RFU)
Measles Virus	4332	4372	3694
Mumps Virus	11377	12621	11454
Rubella Virus	3248	3166	3199

RFU was adjusted to take into account the background.

5 Effects of Dilutions on Caspase 3 Signal

To produce a quantitative assay it is important to detect differences in signal when the sample is diluted to different multiplicity of infections. To address this, measles virus and rubella virus lyophilized samples were diluted 1:2, 1:5 and 1:10 in their respective diluents, and mumps virus was diluted 1:20, 1:40, and 1:80, and the

10 effect on caspase 3 signal was measured. The results are shown in Tables 5 and 6.

TABLE 5

	1:2 Dilution (RFU)	1:5 Dilution (RFU)	1:10 Dilution (RFU)
Measles Virus	8221	3112	1441
Rubella Virus	3184	1721	799

RFU was adjusted to take into account the background.

15

TABLE 6

	1:20 Dilution (RFU)	1:40 Dilution (RFU)	1:80 Dilution (RFU)
Mumps Virus	13681	9524	5061

RFU was adjusted to take into account the background.

activity is affected by the multiplicity of infection used to infect the cells. Because

signal output is affected through dilutions, it is important to have the same multiplicity of infection for direct comparisons between vaccine formulations.

Detection of Differences in Signal Derived from Various Lyophilized Samples

5 The ability of the caspase 3 assay to detect the effect of different sample and storage conditions was measured to determine the suitability of using the caspase 3 assay to measure the stability of viral preparations stored under different conditions. Measles virus was used to infect cells.

10 The data shown in Figure 3 illustrates that the effect of different conditions on viral stability can be measured by measuring caspase 3 activity. Thus, the caspase 3 assay can detect differences in viral potency and stability due to various formulation composition and storage conditions.

Correlation Between the Plaque and Caspase 3 Assays

15 A stability study was performed to examine the correlation between the plaque and caspase 3 assays using measles virus, mumps virus, and rubella virus. The data shown in Figures 4-6 illustrate that as the PFU potency decreased, the corresponding caspase 3 signal also decreased.

20 Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED

1. A method for assaying potency and stability of a virus comprising the steps of:
 - 5 a) contacting a plurality of cells susceptible to caspase 3 induction with said virus; and
 - b) measuring caspase 3 activity as an indication of viral stability.
2. The method of claim 1, wherein said caspase 3 activity is measured using a
10 caspase 3 substrate linked to a fluorimetric or a colorimetric moiety.
3. The method of claim 2, wherein said substrate is the peptide Asp-Glu-Val-Asp.
- 15 4. The method of claim 3, wherein said virus is either measles virus, mumps virus, or rubella virus.
5. The method of claim 4, wherein said plurality of cells is either Vero cells or RK-13 cells.
20
6. The method of claim 3, wherein prior to said step (a) said virus was lyophilized.
7. The method of claim 3, wherein said step (a) and said step (b) are
25 performed at two or more time intervals.
8. The method of claim 3, wherein after said step (a) and prior to said step (b) said cells were frozen and then thawed.
- 30 9. A method of identifying a stabilizing formulation comprising the steps of:
 - a) infecting a first plurality of cells with said virus stored in a first test
strain, and are susceptible to caspase 3 induction, and

b) measuring caspase 3 activity produced from said virus stored in said first and said second test formulation to identify said stabilizing formulation.

5 10. The method of claim 9, wherein said step (a) further comprises contacting a third plurality of cells with said virus stored in a third test formulation, and contacting a fourth plurality of cells with said virus stored in a fourth test formulation, wherein said third and fourth plurality of cells are the same strain as said first and second plurality of cells.

10 11. The method of claim 10, wherein said caspase 3 activity is measured using a caspase 3 substrate linked to a fluorimetric or a colorimetric moiety.

15 12. The method of claim 11, wherein said substrate is the peptide Asp-Glu-Val-Asp.

 13. The method of claim 12, wherein said virus is either measles virus, mumps virus, or rubella virus.

20 14. The method of claim 13, wherein said first, said second, said third, and said fourth plurality of cells is either Vero cells or RK-13 cells.

25 15. The method of claim 12, wherein said virus stored in said first formulation, said second formulation, said third formulation and said fourth formulation was lyophilized.

 16. The method of claim 12, wherein said step (a) and said step (b) are performed at two or more time intervals.

30 17. The method of claim 16, wherein after said step (a) and prior to said step (b) at least one of said first, said second, said third, or said fourth plurality of cells are frozen and thawed.

1/6

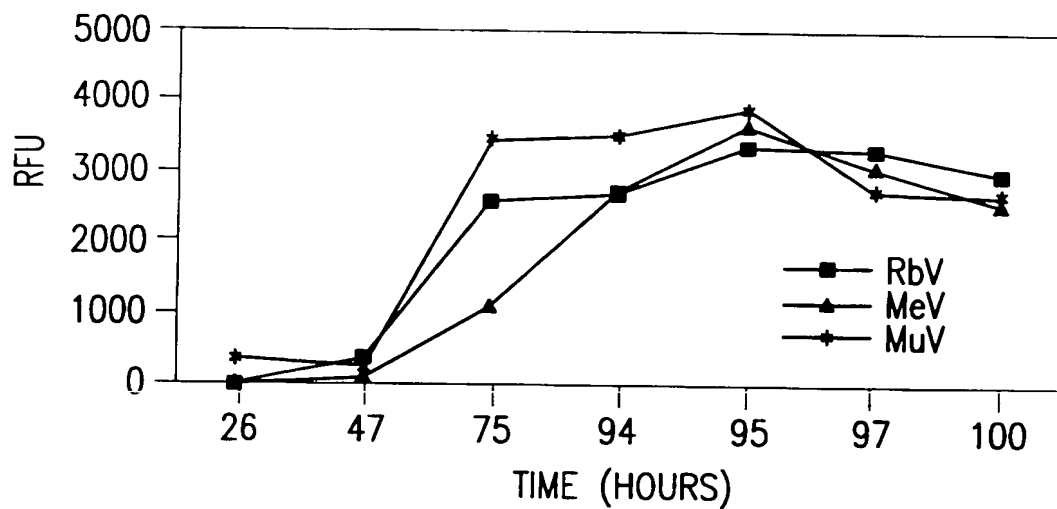


FIG. 1a

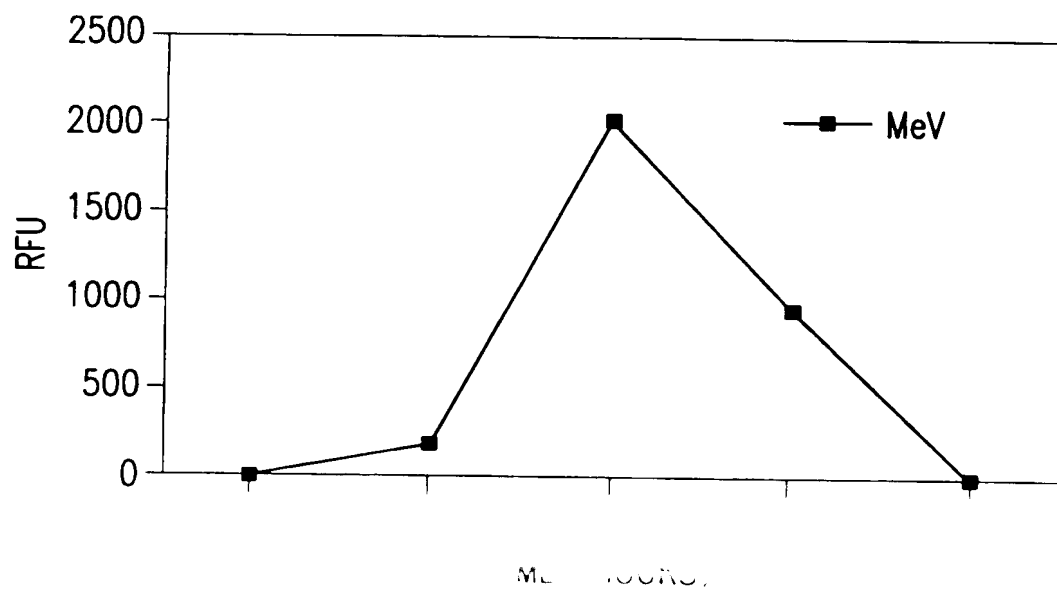


FIG. 1b

2/6

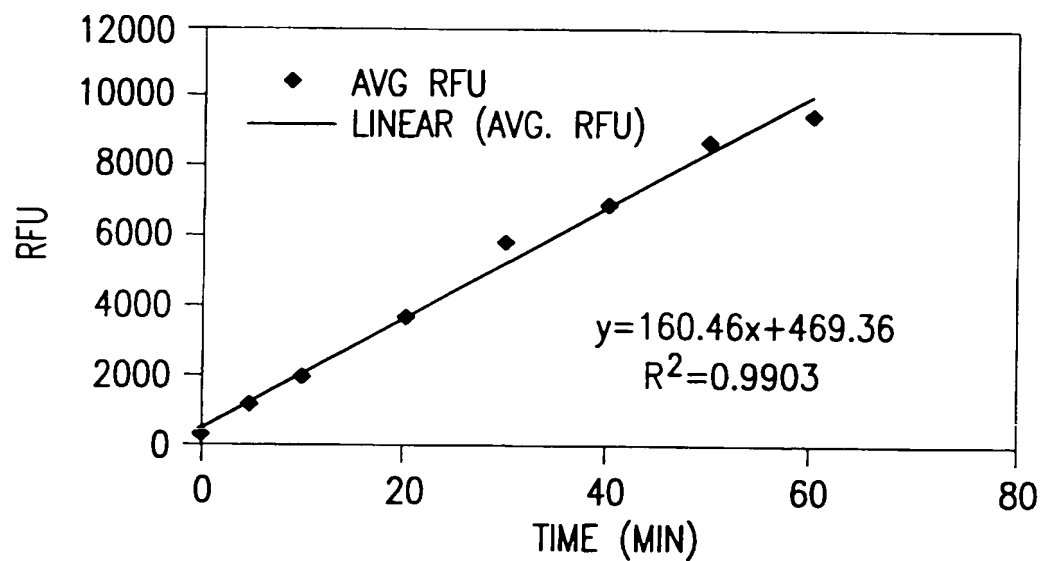


FIG. 2A

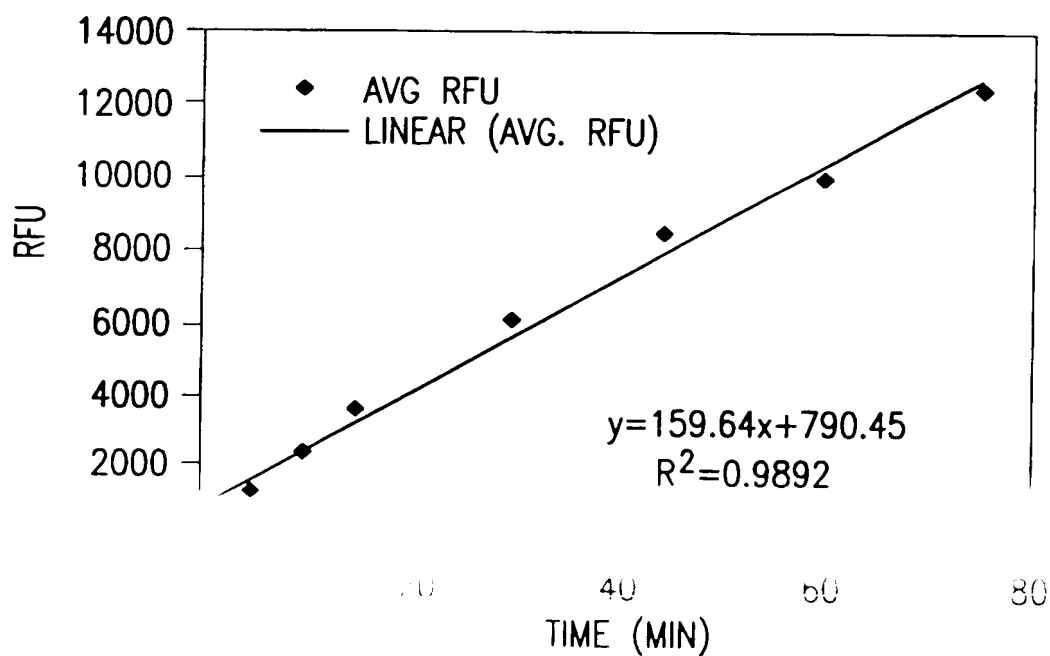


FIG. 2B

3/6

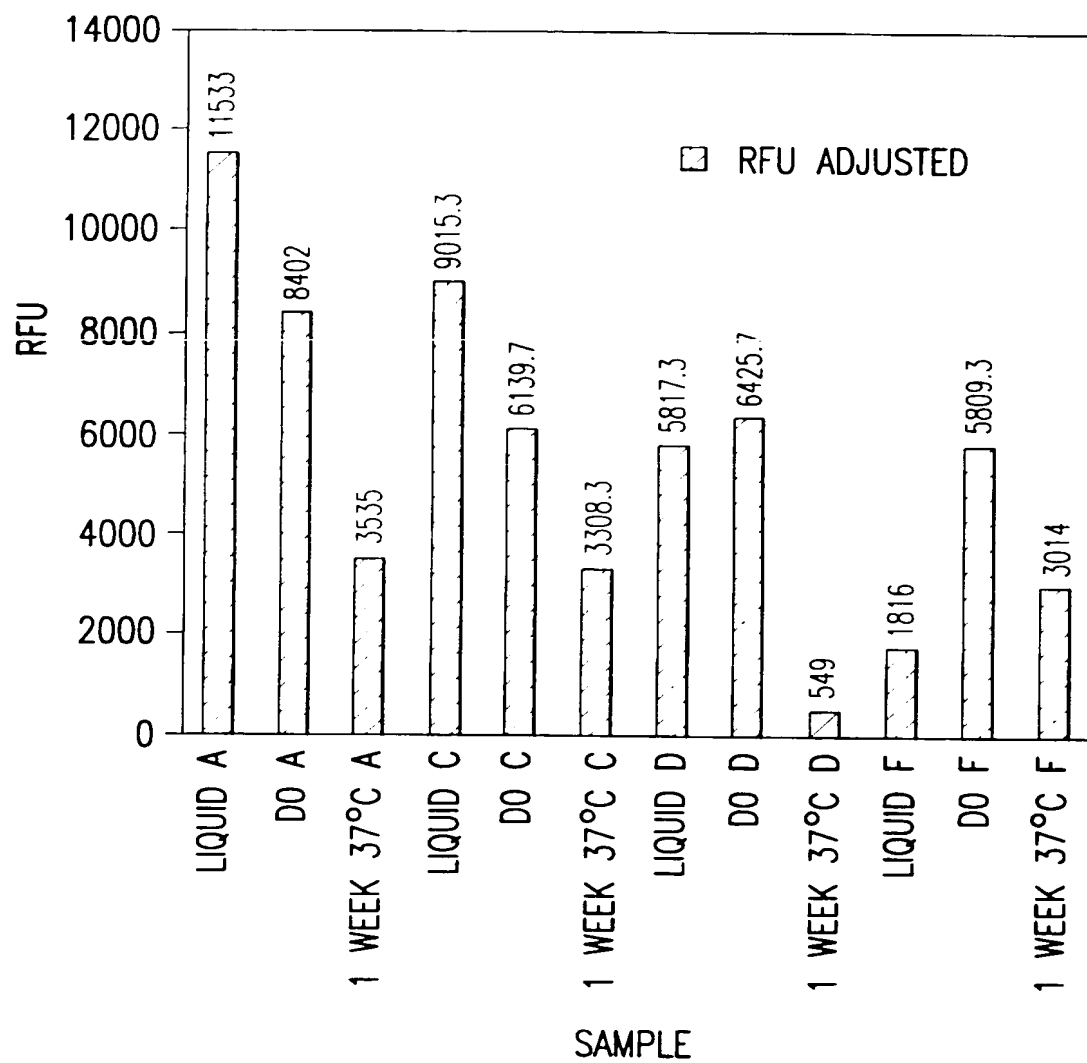


FIG.3

4/6

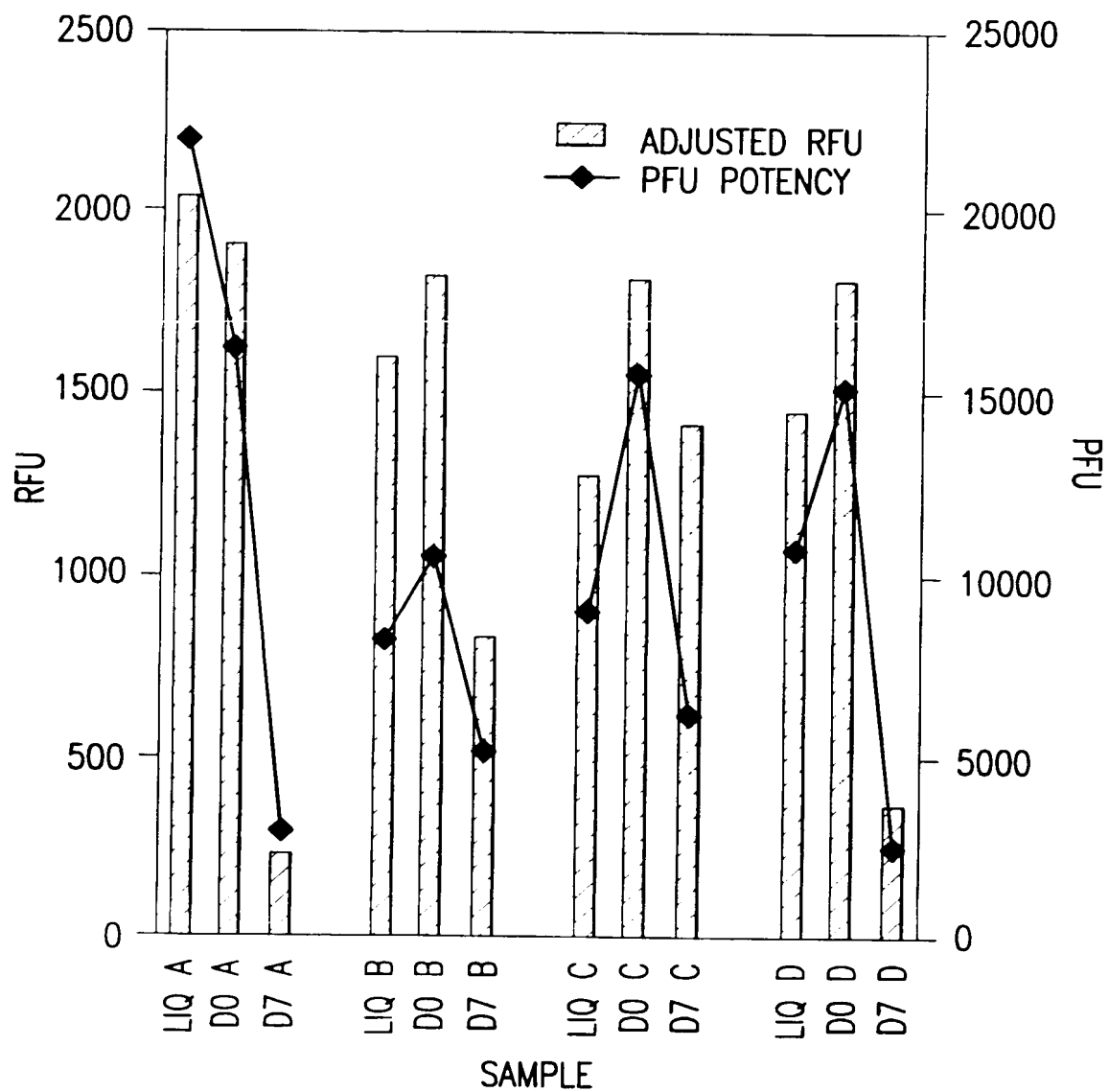


FIG 4

5/6

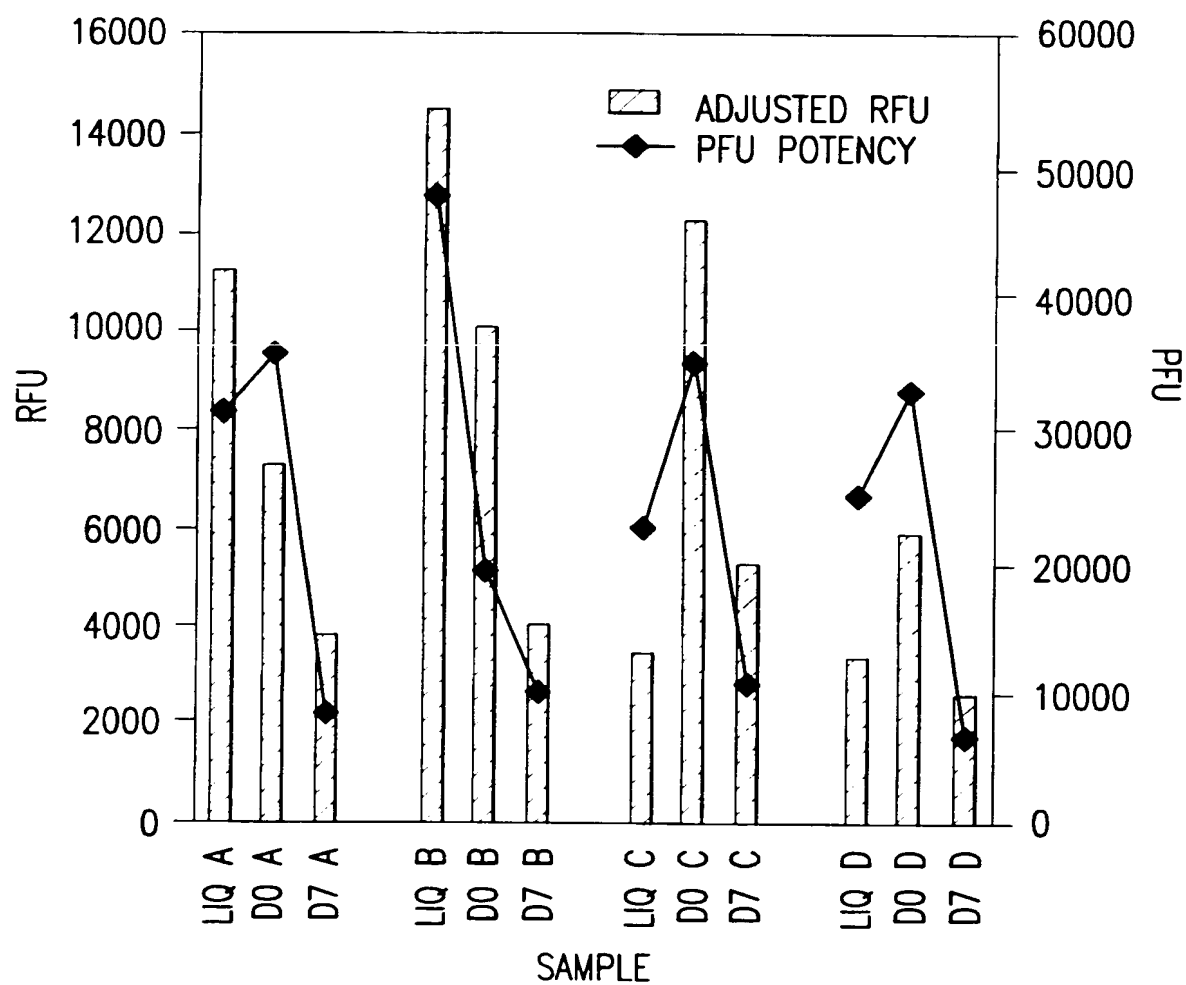


FIG.5

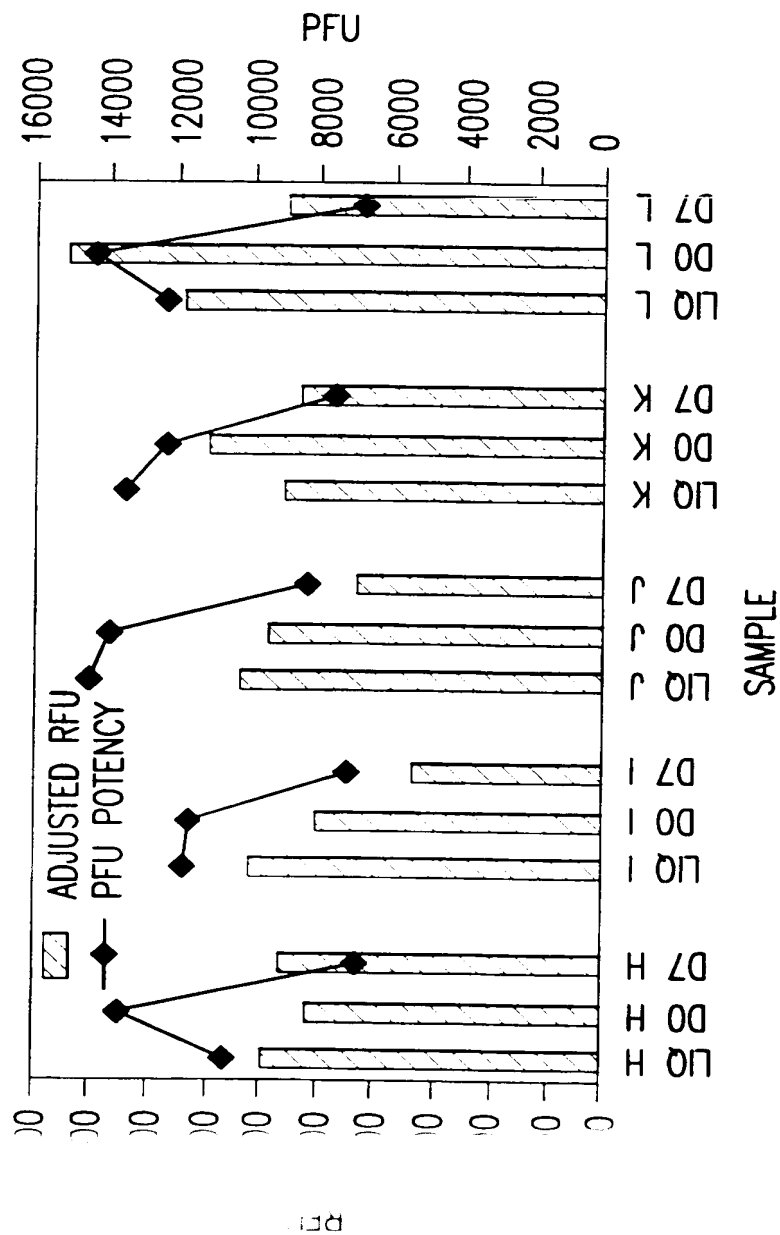


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12638

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/02, 1/70; G01N 33/573; A61K 49/00; A01N 63/00
US CL : 435/5, 7.4, 29; 424/9.2, 93.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.4, 29; 424/9.2, 93.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,976,822 A (LANDRUM et al.) 02 November 1999, see entire document.	1-17
A,P	US 5,972,899 A (ZYCHLINSKY et al.) 26 October 1999, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents and common general knowledge
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
O document referring to an oral disclosure, use, exhibition or other means	

17 JUNE 2000

date of drawing of the international search report

24 JUL 2000

Name and mailing address of the ISA-US
Commissioner of Patents and Trademarks
Box PCT
Washington, DC 20531

Authorized officer

KATHA SCHNEIDER

Facsimile No. (703) 308-5233

Telephone No. (703) 308-1000